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SCIENCE DIRECT
Aquaculture 220 (2003) 165–173
Aquaculture
www.elsevier.com/locate/aqua-online

Streptococcus iniae infection and tissue distribution in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) following inoculation of the gills

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Received 21 August 2002; received in revised form 6 November 2002; accepted 17 November 2002

Abstract

This study was designed to test the possibility that *Streptococcus iniae* enters through the gills and causes infection in hybrid striped bass. To determine the dose response, four groups of fish were inoculated with *S. iniae* via the gills with a dose of 5.0×10^5, 2.6×10^6, 5.0×10^6, or 1.0×10^8 CFU/fish. One group of fish was inoculated with tryptic soy broth (TSB) via the gills to serve as controls. The cumulative percent mortality was 13%, 27%, 100% and 100% for 5.0×10^5, 2.6×10^6, 5.0×10^6 and 1.0×10^8 CFU/fish, respectively. We also examined the tissue dissemination of *S. iniae* at 0.5, 4, 8, 12, 24, 48 and 72 h after experimental gill inoculation. Fish were inoculated with 2.6×10^6 or 5.0×10^6 CFU/fish, which caused low and high mortality, respectively. Within 48 h, fish inoculated with the 2.6×10^6 dose were culture positive on the gill surface, blood of the first and second gill arches, blood of the third and fourth gill arches and the nares. However, for the dose of 5.0×10^6 CFU/fish, *S. iniae* was also isolated from the olfactory, optic and cerebellum regions of the brain, eye, head kidney, trunk kidney, spleen and liver at 48 h. For the 2.6×10^6 dose, *S. iniae* was not isolated until 48 h post-inoculation, but was isolated at 12 h for the 5.0×10^6 dose. The results of this study indicate that *S. iniae* can enter hybrid striped bass through the gills. However, mortality at similar *S. iniae* doses was lower than we previously observed by inoculation of the nares.
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Keywords: Gills; *Streptococcus iniae*; Hybrid striped bass; Infectivity; Distribution; Mortality

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doi:10.1016/S0044-8486(02)00633-6

1. Introduction

Of the 27 recognized species of fish that *Streptococcus* sp. affects, cultured hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) is one of the primary species that is affected in the US aquaculture industry (Stoffregen et al., 1996; Bowser et al., 1998; Shoemaker et al., 2001). Streptococcal disease has been shown to occur after direct contact with fish carrying the bacteria (Kitao, 1993). The infection can be spread to healthy fish by ingestion of fish food made from streptococcal infected fish (Minami, 1979). Injured skin was also reported to be a portal of entry into the fish (Rasheed and Plumb, 1984). Additionally, the disease has been established by injection (intraperitoneal or intramuscular) (Stoffregen et al., 1996). Successful entry of *Streptococcus iniae* through the olfactory organ and subsequent infection has been demonstrated (Evans et al., 2000). In channel catfish (*Ictalurus punctatus*) another bacterial pathogen, *Edwardsiella ictaluri*, has been shown to enter via the gills (Nusbaum and Morrison, 1996). This route of entry (i.e., the gills) has not been demonstrated for *S. iniae*; therefore, the objective of this study was to determine if *S. iniae* was capable of infecting hybrid striped bass following experimental inoculation of the gills.

2. Methods and materials

2.1. Fish

Hybrid striped bass (*M. chrysops* × *M. saxatilis*) were purchased from Keo Fish Farms (Keo, Arkansas, USA) and were maintained in the USDA-ARS facilities. The hybrid striped bass had an average weight of 23.8 g and an average length of 131.1 mm and were culture negative for *S. iniae* by standard methods (Shoemaker and Klesius, 1997) prior to the experiment. Fish were acclimated for 5 days in a 57-l glass aquaria supplied with flow through water at 0.5 l/min. A light and dark period of 12:12 h was maintained and aeration was supplied through air stones. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, MO).

2.2. Water quality evaluation

The dissolved oxygen, temperature, pH, salinity, hardness, ammonia and nitrites were maintained within acceptable ranges. During the experiment, the mean ± standard deviation of dissolved oxygen was 5.8 ± 0.5 mg/l, temperature was 26.2 ± 0.8 °C, pH was 7.1 ± 0.3, salinity was 0.1 ± 0.0% and hardness was 110 ± 10 mg/l ($CaCO_3$). Ammonia and nitrite concentrations (mg/l) were below the detection limit for the experiments.

2.3. Bacteria

S. iniae strain ARS-98-60 was used to infect the fish. The isolate was identified as *S. iniae* according to tests described by Shoemaker and Klesius (1997) based on the original description of *S. iniae* by Pier and Madin (1976). The isolate was grown in tryptic soy broth (TSB) for 24 h at 27 °C, and then the culture was frozen at −70 °C in 0.2 ml aliquots with 15% glycerol (v/v). The infectious isolate used in this study was prepared by inoculating 250 ml of TSB in a 500-ml culture flask with the isolate. Following incubation without shaking for 24 h at 27 °C, the culture was adjusted spectrophotometrically to an optical density of 1.0 at 540 nm to give a concentration of 1.0×10^8 CFU/ml. The doses of *S. iniae* used in this study were then prepared by dilution in TSB, or concentrated via centrifugation prior to the experiment, and the CFU was confirmed by plate counts (Evans et al., 2001).

2.4. Experimental gill inoculation

Hybrid striped bass were netted, placed into a holding tank, and anesthetized with MS-222 (Argent, Redmond, WA, USA) (Evans et al., 2000). Each fish was then picked up and inoculated with either 250 or 500 μl per gill chamber of the appropriate CFU of a TSB suspension of *S. iniae*. The gill chambers of each fish were slowly instilled with the appropriate aliquot of the culture suspension or control TSB using a micropipet. For each dose, half of the total amount of the suspension was instilled over all gill arches in one opercular cavity, and this process was repeated for the opposite opercular cavity to create the appropriate dose. For both the distribution study and the mortality and behavior study, both gill chambers were inoculated. The fish were placed on moist paper towels for 2 min to allow the bacteria to attach to the gills. The fish were then placed back into their respective tanks and monitored for 5–10 min.

2.5. Mortality

Four groups of 15 fish were inoculated via the gills with *S. iniae* with a dose of 5.0×10^5, 2.6×10^6, 5.0×10^6, or 1.0×10^8 CFU/fish, respectively. The fish were monitored for mortalities and clinical signs of *S. iniae* infection for 14 days. Freshly dead fish were examined microbiologically as described by Shoemaker and Klesius (1997). Fish were considered culture positive if any *S. iniae* colonies were identified morphologically and biochemically from the blood agar plates.

2.6. Tissue distribution

Two groups of 30 fish each were inoculated with 2.6×10^6 and 5.0×10^6 CFU/fish. Two groups of 15 fish each were inoculated with TSB only to serve as the control fish. Three *S. iniae* inoculated fish were randomly selected out of trial aquaria at 0.5, 4, 8, 12, 24, 48 and 72 h to determine the tissue dissemination of *S. iniae*. Samples were taken from the gill surface, blood of the first and second gill arches, blood of the third and fourth gill arches, nares, olfactory, optic and cerebellum regions of the brain, eye, head kidney, trunk kidney, spleen, liver and intestine using a 1-μl bacterial loop (VWR, West Chester, PA, USA). All samples were collected by inserting a bacterial loop into the designated organ, tissue or blood. To estimate the CFU in a 1-μl or 1-μg sample, colony counts on a 4-cm streak on sheep blood agar plates were performed visually using a colony counter (Evans et al., 2001). The plates were incubated at 27 °C for 24 h. Any streak with greater than 100

CFU was recorded as 100 CFU, because accurate counts could not be made over 100 CFU. The results were expressed as the estimated mean CFU/1-μl sample. For each time interval, at least one colony was bacteriologically and biochemically identified as *S. iniae* according to tests described by Shoemaker and Klesius (1997). None of the control fish that received TSB tested positive for *S. iniae*. Data for this experiment were analyzed by one-way analysis of variance (SAS Institute, 2000). Duncan's multiple range tests were used to determine significant differences in CFU counts between organs at each time period and in CFU counts for each organ over time. All data were considered significant at $p < 0.05$.

3. Results

3.1. Mortality

Mortality due to *S. iniae* occurred on day 2 when two fish that were inoculated with a dose of 5.0×10^6 CFU died and were found to be culture positive for *S. iniae*. Fish inoculated with the highest dose (1.0×10^8) began dying on day 3. The first mortality for the 5.0×10^5 and 2.6×10^6 doses were observed at day 7. However, most fish administered the lower doses survived for the 14-day trial and exhibited signs of *S. iniae* disease (i.e., eye opacity and dark coloration). The cumulative percent mortality was 13%, 27%, 100% and 100% for 5.0×10^5, 2.6×10^6, 5.0×10^6 and 1.0×10^8 doses, respectively (Table 1).

3.2. Tissue distribution

The first tissues that were culture positive for *S. iniae* at the 2.6×10^6 CFU/fish dose occurred within 48 h and were the gills, blood from the first and second gill arches, blood from the third and fourth gill arches and the nares (Table 2A). At 72 h, *S. iniae* was isolated from the olfactory and cerebellum lobes of the brain and the liver. No more than a mean of 4.3 CFU of *S. iniae*/μg of tissue or μl of blood was cultured from the fish administered the 2.6×10^6 CFU dose. Statistical analysis of the CFU/μl or μg values of all tissues at 48 h demonstrated the blood of the first and second gill arches, blood of the third and fourth gill arches and the nares were significantly ($p < 0.05$) higher than the other tissues sampled (Table 2A). Blood of the first and second gill arches yielded the highest

Table 1
Cumulative mortality of *S. iniae*-infected hybrid striped bass at 14 days post-challenge following gill inoculation

Dose (CFU/fish)	Number of fish per aquaria	Number of dead per number total*	Percent mortality (%)
5.0×10^5	15	2:15	13
2.6×10^6	15	4:15	27
5.0×10^6	15	15:15	100
1.0×10^8	15	15:15	100

* All dead fish were culture positive on blood agar.

Table 2
Gill inoculation of *S. iniae* and its tissue/organ dissemination in hybrid striped bass[1]

Time (h)	Colony-forming units/μg or μl[A]												
	Gill	Blood[B] (first and second)	Blood[C] (third and fourth)	Nare	Olfactory lobe	Optic lobe	Cerebellum	Eye	Head kidney	Caudal kidney	Spleen	Liver	Intestine
(A) Dose = 2.6 × 10⁶ CFU/fish													
48**	1 ± 1.7^{c}	4.3 ± 2.1^{a}	2.7 ± 1.2^{b}	1.7 ± 1.5^{bc}									
72	1 ± 1.7^{a}	2.7 ± 3.1^{a}	0.7 ± 0.6^{a}	1 ± 1.7^{a}	0.3 ± 0.6^{a}		0.3 ± 0.6^{a}					4.0 ± 6.9^{a}	
(B) Dose = 5.0 × 10⁶ CFU/fish													
12***	1 ± 1.7^{a}	3.3 ± 4.2^{a}	5.0 ± 8.7^{a}	3.0 ± 2.6^{a}	1.7 ± 2.1^{a}	0.3 ± 0.6^{a}	1 ± 1.0^{a}	1.7 ± 1.5^{a}	0 ± 0.0^{a}	4.7 ± 4.2^{a}	1.3 ± 2.3^{a}	1.3 ± 2.3^{a}	1 ± 1.7^{a}
24													
48	1 ± 1.7^{a}	19 ± 32.9^{a}	6.0 ± 10.4^{a}	66.7 ± 57.7^{a}	66.7 ± 57.7^{a}	33.3 ± 57.7^{a}	33.3 ± 57.7^{a}	14 ± 19.3^{a}	31 ± 53.7^{a}	21.3 ± 37.0^{a}	33.3 ± 57.7^{a}	25.7 ± 42.7^{a}	
72	37 ± 29.2^{abc}	33.3 ± 57.7^{bc}	33.3 ± 57.7^{bc}	62 ± 54.1^{abc}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	94.3 ± 9.8^{ab}	100 ± 0.0^{a}	74 ± 45.0^{abc}	87 ± 22.5^{ab}	100 ± 0.0^{a}	21.7 ± 27.1^{c}

[1] Mean *S. iniae* colony-forming units (CFU) ± standard deviation. Mean CFU values are compared across rows (between tissues). Means with the same letter per row are not significantly different ($p < 0.05$).

[A] $N = 3$ for all times and Blank space denotes 0.0 ± 0.0 CFU/μg.

[B] Denotes blood from the first and second gill arches.

[C] Denotes blood from the third and fourth gill arches.

** *S. iniae* was not isolated before 48 h.

*** *S. iniae* was not isolated before 12 h.

Table 3
Gill inoculation of *S. iniae* and its tissue/organ dissemination in hybrid striped bass[1]

Time (h)	Colony-forming units/μg or μl[A]												
	Gill	Blood[B] (first and second)	Blood[C] (third and fourth)	Nare	Olfactory lobe	Optic lobe	Cerebellum	Eye	Head kidney	Caudal kidney	Spleen	Liver	Intestine
(A) Dose = 2.6 × 10⁶ CFU/fish													
48**	1 ± 1.7^{a}	4.3 ± 2.1^{a}	2.7 ± 1.2^{a}	1.7 ± 1.5^{a}									
72	1 ± 1.7^{a}	2.7 ± 3.1^{ab}	0.7 ± 0.6^{b}	1 ± 1.7^{a}	0.3 ± 0.6^{a}		0.3 ± 0.6^{a}					4.0 ± 6.9^{a}	
(B) Dose = 5.0 × 10⁶ CFU/fish													
12***	1 ± 1.7^{c}	3.3 ± 4.2^{a}	5.0 ± 8.7^{a}	3.0 ± 2.6^{a}	1.7 ± 2.1^{b}	0.3 ± 0.6^{c}	1 ± 1.0^{b}	1.7 ± 1.5^{b}		4.7 ± 4.2^{b}	1.3 ± 2.3^{b}	1.3 ± 2.3^{c}	1 ± 1.7^{b}
24													
48	1 ± 1.7^{c}	19 ± 32.9^{a}	6.0 ± 10.4^{a}	66.7 ± 57.7^{a}	66.7 ± 57.7^{a}	33.3 ± 57.7^{b}	33.3 ± 57.7^{b}	14 ± 19.3^{b}	31 ± 53.7^{b}	21.3 ± 37.0^{b}	33.3 ± 57.7^{b}	25.7 ± 42.7^{bc}	
72	37 ± 29.2^{b}	33.3 ± 57.7^{a}	33.3 ± 57.7^{a}	62 ± 54.1^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	94.3 ± 9.8^{a}	100 ± 0.0^{a}	74 ± 45.0^{a}	87 ± 22.5^{a}	100 ± 0.0^{a}	21.7 ± 27.1^{b}

[1] Mean *S. iniae* colony-forming units (CFU) ± standard deviation. Mean CFU values are compared down the columns (each organ over time). Means with the same letter per column are not significantly different ($p < 0.05$).

[A] $N = 3$ for all times and Blank space denotes 0.0 ± 0.0 CFU/μg.

[B] Denotes blood from the first and second gill arches.

[C] Denotes blood from the third and fourth gill arches.

** *S. iniae* was not isolated before 48 h.

*** *S. iniae* was not isolated before 12 h.

values of CFU. However, by 72 h, none of the tissues yielding *S. iniae* were significantly different.

Mean CFU were not significantly different for any tissues for the first 24 h for the 5.0×10^6 dose, because all tissues were culture negative. *S. iniae* was cultured in low numbers from every tissue except the head kidney at 12 h for the 5.0×10^6 dose (Table 2B). *S. iniae* was not recovered from any tissues sampled at 24 h. At 48 h, all tissues were culture positive, except the intestine. At 72 h the olfactory, optic and cerebellum lobes, head kidney and liver all averaged over 100 CFU of *S. iniae*/μg of tissue and were significantly higher ($p < 0.05$) than all other tissues (Table 2B). The CFU in individual tissues compared over time showed that the gills, olfactory, optic and cerebellum lobes, eye, head and trunk kidney, spleen and liver all became significantly ($p < 0.05$) higher at 72 h than at 12 h in fish given 5.0×10^6 CFU (Table 3B).

4. Discussion

Significantly ($p < 0.05$) higher *S. iniae* CFU counts were not observed until after 24 h for either the 2.6×10^6 or 5.0×10^6 dose. For fish administered 2.6×10^6, the highest CFU counts were found in the gills, blood from the first and second and the third and fourth gill arches. Although this could be perceived as residual bacteria from the gill inoculation, the fact that *S. iniae* was not recovered from these areas until 48 h post-inoculation suggests that replication occurred. Because *S. iniae* was recovered from the brain and other organs, we have shown that *S. iniae* is able to penetrate the blood–brain barrier and can disseminate to other organs and tissues following inoculation via the gills. For fish administered 5.0×10^6 CFU/fish dose, *S. iniae* was isolated from all but one tissue sampled (head kidney) as early as 12 h. This suggests that at a higher dose, *S. iniae* is probably more likely to cross the membrane of the gill lamellae and is more readily disseminated throughout other tissues. At 48 h, replication in most tissues had taken place.

The distribution and spread of *S. iniae* within tissues may differ depending on the dose of *S. iniae*. At the lower dose, 2.6×10^6, only two tissues, the blood of the first and second gill arches and the third and fourth gill arches, ever became significantly different ($p < 0.05$) when each organ was compared over time (Table 3A). In contrast, the blood of the first and second gill arches, blood from the third and fourth gill arches, along with the nares and intestine were the only sites that did not become significantly different for the 5.0×10^6 dose when comparing each organ over time (Table 3B). These results show that the *S. iniae* infection caused by the 5.0×10^6 dose became septicemic. Since the infection had disseminated to the intestinal tract and was replicating, as indicated by increased CFUs, we believe that *S. iniae* may be released into the water through fecal waste.

The gills and nares have been suggested as portals of entry for both *S. iniae* and *E. ictaluri*. Evans et al. (2001) were able to isolate *S. iniae* from nine tissues in hybrid striped bass within 18 h of inoculation of the nares. We were able to isolate *S. iniae* from 12 tissues when hybrid striped bass were inoculated via the gills with *S. iniae* at a dose of 5×10^6. Even though we were able to recover *S. iniae* following gill inoculation, it appears that to achieve the same rate of mortality and level of infection that can be achieved by nare inoculation, a higher dose must be used. Morrison and Plumb (1994) found that during the initial stages of infection, the olfactory organ of channel catfish was vulnerable to *E. ictaluri* infection following micropippetting of 1×10^6 CFU into the anterior incurrent nares. Nusbaum and Morrison (1996) bathed channel catfish for 1 h in water containing 5.6×10^6 *E. ictaluri*/ml labeled with [^{35}S] methionine. They suggested that *E. ictaluri* entered through the gill, and therefore, the gills should be considered a route of infection. However, no fish in their study became sick or died. McNulty et al. (unpublished data) have observed that *E. ictaluri* can infect channel catfish by pippetting a broth containing *E. ictaluri* over the gills. However, to achieve infection and significant mortality, a dose of 2×10^9 CFU was required.

This study demonstrates that *S. iniae* can invade gill filament epithelium, travel via the blood, infect the brain and disseminate throughout the body. However, low mortality (13.33%) was observed by inoculating the gills with 5×10^5 CFU/hybrid striped bass in this study. Evans et al. (2000), who inoculated via the nares, produced 76% mortality using 2.6×10^4 CFUs and were able to establish infection in hybrid striped bass at levels as low as 4.8×10^3 CFU with mortality at 26%. In another study, Evans et al. (2001) showed increased bacterial counts in all tissues by 18 h following inoculation of the nares with 1×10^5 CFU. In our current study using 20-fold more bacteria (2.6×10^6 CFU), culture-positive organs did not become readily apparent until 48 h. The gills may be a viable route of *S. iniae* entry; however, the bacterial load needed to establish infection appears to be greater than required by the nare route of *S. iniae* entry.

Acknowledgements

We would like to thank Dr. John Plumb and Dr. John Grizzle for reviewing this manuscript.

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